



## Bioactive properties and functional constituents of *Hypericum androsaemum* L.: A focus on the phenolic profile

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### ABSTRACT

*Hypericum androsaemum* L. ethanol:water extract acted as a lipid peroxidation inhibitor and free radical scavenger. A marked inhibition of the growth of breast, lung, cervical and hepatocellular human carcinoma cell lines was also observed, whereas no toxicity was shown against non-tumor porcine liver cells (>400 µg/mL). The extract was also effective in inhibiting nitric oxide production, as an indicator of the anti-inflammatory potential. The anti-*Candida* effects varied among different strains of the same species, *C. glabrata* and *C. tropicalis* being the most sensible species with an effect directly related with the extract concentrations tested. A significant anti-biofilm formation potential was also observed, namely for *C. glabrata* and *C. tropicalis* (biofilm reduction >90%). 5-*O*-Caffeoylquinic and 3-*O*-caffeoylquinic acids were the most abundant phenolic compounds identified in the extract, and might be related with the observed bioactive effects. Nevertheless, future studies should be carried out to obtain dose-response curves of the isolated active compounds, in order to perform further pre-clinically testing to quantify the presence of the most active compounds in the extract.

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## 1. Introduction

Medicinal plants have been used over years for multiple purposes, and have increasingly attracted the interest of researchers in order to evaluate their contributes to health maintenance and disease's prevention (Murray and Gramercy Books, 2004). Several studies have been carried out, but numerous active principles directly responsible for the observed bioactive properties remain unknown, while in other cases the mechanism of action is not fully understood.

*Hypericum* species (Hypericaceae family; nearly about 450 species) are examples of these plants, being *Hypericum perforatum* L. the most widely known and with attributed anti-depressive, anti-inflammatory, antitumor, antimicrobial, antioxidant and antiviral benefits (Nogueira et al., 2013; Rainha, Lima, & Baptista, 2011; Ramalhete et al., 2016). However, based on recent findings, other *Hypericum* species have

also revealed very interesting functional properties. *Hypericum androsaemum* L., commonly known as sweet-amber, has a popular use as cholagogue, hepatoprotector, nephroprotective and diuretic (Guedes, Amorim, Vicente, Ramos, & Fernandes-Ferreira, 2003). In fact, *Hypericum* species are also used for culinary and cosmetic purposes and, in particular, the use of *H. androsaemum* as a spice, herbal tea and even its external application in wounds and burns (due to its healing potential) is also highly recommended (Mazandarani, Yassaghi, Rezaei, Mansourian, & Ghaemi, 2007).

The antioxidant potential of a *H. androsaemum* aqueous extract prepared from the whole plant (Hernandez, Falé, Araújo, & Serralheiro, 2010; Ramos, Marques, Fernandes-Ferreira, & Pereira-Wilson, 2013), aerial parts (Rainha et al., 2011) and leaves (Valentão et al., 2002) has been described. In addition, the hepatoprotective effects of aqueous obtained from leaves have been reported, and directly correlated with their antioxidant capacity (Valentão et al., 2004). The antitumor potential of *H. androsaemum* aqueous extracts was also studied (Xavier, Lima, Fernandes-Ferreira, & Pereira-Wilson, 2012), through the evaluation of their anti-proliferative and pro-apoptotic effects in human colorectal cancer cells. It is important to highlight that no reports have been

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found evaluating its anti-inflammatory potential, which is of utmost importance once inflammation is not only a consequence of the free radicals overproduction, but also a cause of numerous disorders, such as neurodegenerative and cardiovascular diseases, and even cancer (Valko et al., 2007). Regarding antimicrobial effects, those were only described for ethanol extracts obtained from the aerial parts (Nogueira et al., 2013). In fact, opportunistic fungal infections constitute a serious threat to human health and wellbeing (Lott, Fundyga, Kuykendall, & Arnold, 2005), and for many years, *Candida albicans* has been designated as the most widespread involved opportunistic pathogenic yeast (Guinea, 2014). However, other non-*C. albicans* *Candida* (NCAC) species, such as *C. glabrata*, *C. tropicalis* and *C. parapsilosis*, have been already and increasingly isolated mainly due to the indiscriminate prescription of antifungal agents (Estivill, Arias, Torres-Lana, Carrillo-Munoz, & Arevalo, 2011; Gacser, Schafer, Nosanchuk, Salomon, & Nosanchuk, 2007; Kuhn, Chandra, Mukherjee, & Ghannoum, 2002). More interestingly is that the pathogenesis of candidiasis is common to all *Candida* species and facilitated by several virulent factors (Silva et al., 2012). The major virulence factor of *Candida* species is its ability to adapt to a variety of different habitats, with the consequent formation of surface-attached microbial communities known as biofilms (Chandra et al., 2001; Douglas, 2003; Ganguly & Mitchell, 2011). Therefore, the presence of persists in the *Candida* biofilm greatly tolerates high dose of fungicidal (Lewis, 2007), which complicates the clinical therapy for the nosocomial infections (Moran, Grussemeyer, Spalding, Benjamin, & Reed, 2010). Thus, the assessment of the biofilm formation inhibitory potential of *H. androsaemum* may be conceived as a promissory antifungal therapy.

The composition of *H. androsaemum* in terms of phenolic compounds has been analyzed by some authors, namely in aqueous (Valentão et al., 2002; Valentão et al., 2004) and methanol (Porzel, Farag, Müllbradt, & Wessjohann, 2014; Valentão et al., 2003) extracts from leaves and aerial parts (Rainha et al., 2011; Ramalheite et al., 2016). Nevertheless, there are no studies reporting different bioactive properties (antioxidant, cytotoxic, anti-inflammatory and anti-*Candida* properties) in a specific extract of *H. androsaemum* (ethanol: water, 80:20, v/v), and relating them with its phenolic composition, which was carried out in the present work. The biological effects to be tested were selected based on the fact that high levels of oxidative stress promotes inflammation, as also immune system weakening, cell damages, and consequently enhances the growth of cancerous cells. Otherwise, inflammatory, infectious and also carcinogenic processes involve free radicals overproduction, which needs to be controlled. Furthermore, numerous bioactive ingredients derived from plants and other natural matrices have been increasingly applied as food ingredients, not only for organoleptic purposes but also for nutritional and even health-enhancing purposes. Therefore, the study of upcoming natural matrices may be also conceived not only as a source of effective and upcoming therapies, but also a way to discover promissory bioactive molecules for food industry and even pharmaceutical purposes.

## 2. Material and methods

### 2.1. Plant material and preparation of the ethanol:water extract

Aerial parts of *Hypericum androsaemum* L., namely leaves, flowers and inflorescences were provided by "Cantinho das Aromáticas", an organic and certified farm from Vila Nova de Gaia, Portugal and the botanical identification was confirmed by the botanist, Dr. Carlos Aguiar from School of Agriculture at the Polytechnic Institute of Bragança, Portugal.

The dried samples were reduced to powder and submitted to the following extraction procedure, at room temperature ( $\sim 25^\circ\text{C}$ ), in three independent experiments: 4 g were stirred with 30 mL of ethanol: water (80:20, v/v) for 1 h, filtered and re-extracted twice in the same conditions. Ethanol was removed in a rotary evaporator (Büchi R-210, Flawil, Switzerland), while water was removed by lyophilisation ( $-48^\circ\text{C}$  and 0.100 mbar, during 2–3 days, FreeZone 4.5, Labconco, Kansas City, MO,

USA). Stock solutions of the extract were prepared with water: 5 mg/mL (for antioxidant activity assays), 8 mg/mL (for antitumor and anti-inflammatory activity assays) and 50 mg/mL (for anti-*Candida* activity assays), from which several dilutions were performed. Different concentrations were used for each *in vitro* assay due to the different parameters of sensitivity, specificity and reliability of the techniques. For phenolic compounds analysis the extract (2.5 mg/mL) was filtered through a  $0.45\ \mu\text{m}$  Whatman syringe filter and transferred to an amber HPLC vial for chromatographic injection.

### 2.2. Standards and reagents

HPLC grade acetonitrile 99.9% and analytical grade purity ethanol were from Fisher Scientific (Lisbon, Portugal). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Formic and acetic acids were purchased from Prolabo (VWR International, France). Trolox (6-hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid), sulforhodamine B, trypan blue, trichloroacetic acid (TCA), Tris, 3-(N-Morpholino)propanesulfonic acid (MOPS) and RPMI 1640 medium were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sabouraud Dextrose Broth (SDB) and Sabouraud Dextrose Agar (SDA) were from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium, Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), were purchased from Gibco Invitrogen Life Technologies (California, USA). RAW264.7 cells were purchased from ECACC ("European Collection of Animal Cell Culture") (Salisbury, UK), lipopolysaccharide (LPS) from Sigma and DMEM medium from HyClone. The Griess Reagent System Kit was purchased from Promega, and dexamethasone from Sigma. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

### 2.3. Evaluation of the antioxidant activity

Scavenging effects on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals (RSA): an ELX800 microplate Reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA) was used to evaluate and to calculate the percentage of DPPH discolouration through the formula:  $[(A_{\text{DPPH}} - A_s) / A_{\text{DPPH}}] \times 100$ , where  $A_s$  is the absorbance of the solution containing the extract solution at 515 nm, and  $A_{\text{DPPH}}$  is the absorbance of the DPPH solution (Martins, Barros, Dueñas, Santos-Buelga, & Ferreira, 2015).

Reducing power (measured by ferricyanide Prussian blue assay) (RP): through this assay the capacity to convert  $\text{Fe}^{3+}$  into  $\text{Fe}^{2+}$  was assessed, measuring the absorbance at 690 nm in the microplate Reader above mentioned (Martins et al., 2015).

$\beta$ -carotene bleaching inhibition (CBI): was determined by the  $\beta$ -carotene/linoleate assay. The absorbance at zero time and after 2 h was measured at 470 nm in a spectrophotometer (AnalytikJena, Jena, Germany). The neutralization of linoleate free radicals avoids  $\beta$ -carotene bleaching, which was calculated by the formula:  $\beta$ -carotene absorbance after 2 h of assay / initial absorbance  $\times 100$  (Martins et al., 2015).

Lipid peroxidation inhibition (LPI): this assay was based on the ability to decrease thiobarbituric acid reactive substances (TBARS) generation in porcine brain cell homogenates, which was used as a direct indicator of the antioxidant potential of extract. The color intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) adduct was measured by its absorbance at 532 nm and the inhibition ratio (%) was calculated using the following formula:  $[(A - B) / A] \times 100\%$ , where A and B were the absorbance of the control and the extract solution, respectively (Martins et al., 2015).

For all antioxidant assays three independent experiments were carried out and the results were expressed as EC<sub>50</sub> values, which correspond to the extract concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

## 2.4. Evaluation of the cytotoxic activity

### 2.4.1. In human tumor cell lines

In order to perform a complete *in vitro* cytotoxicity screening, four human tumor cell lines were selected: HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer), being among the most representative carcinoma cell lines. In all cases the cell lines were plated in 96-well plates, with a final density of  $1.0 \times 10^4$  cells/well, and were allowed to attach for 24 h. Then, various extract concentrations were added to the cells and incubated during 48 h. Afterwards, cold trichloroacetic acid (TCA 10%, 100  $\mu$ L) was used in order to bind the adherent cells and further incubated for 60 min at 4 °C. After the incubation period, the plates were washed with deionised water and dried, and sulforhodamine B solution (SRB 0.1% in 1% acetic acid, 100  $\mu$ L) was added to each plate well and incubated for 30 min at room temperature. Then, the plates were washed with acetic acid (1%) in order to remove the unbound SRB and air dried, the bounded SRB was solubilised with Tris (10 mM, 200  $\mu$ L) and the absorbance was measured at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA) (Abreu et al., 2011).

### 2.4.2. In non-tumor porcine liver cells (PLP2)

Liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL of penicillin and 100  $\mu$ g/mL of streptomycin, and then divided into  $1 \times 1 \text{ mm}^3$  explants. Some of these explants were placed in 25 cm<sup>3</sup> tissue flasks, containing DMEM and supplemented with 10% fetal bovine serum, 2 mM of non-essential amino acids, 100 U/mL of penicillin and 100 mg/mL of streptomycin, and then incubated at 37 °C with a humidified atmosphere containing 5% CO<sub>2</sub>. This medium was changed every 2 days, and the cells cultivation continued under direct monitoring every 2–3 days using a phase contrast microscope. Prior to confluence, cells were sub-cultured and plated in 96-well plates, with a final density of  $1.0 \times 10^4$  cells/well and cultivated in DMEM medium, containing 10% FBS, 100 U/mL of penicillin and 100  $\mu$ g/mL of streptomycin (Abreu et al., 2011). Then, cells were treated for 48 h with the different diluted sample solutions. Three independent experiments were carried out and the results were expressed in GI<sub>50</sub> values, i.e. the extract concentration that inhibited 50% of the net cell growth. Ellipticine was used as positive control.

## 2.5. Evaluation of the anti-inflammatory activity

The mouse macrophage-like cell line RAW264.7 was cultured in DMEM medium, supplemented with 10% heat-inactivated fetal bovine serum and L-glutamine, at 37 °C under 5% CO<sub>2</sub>, in humidified air. Cells were detached with a cell scraper, the experimental cell density established in  $5 \times 10^5$  cells/mL and the proportion of cell dead lesser than 1%, according to Trypan blue dye exclusion tests. Then, cells were seeded in 96-well plates at 150,000 cells/well and their attachment to the plate allowed overnight. Afterwards, cells were treated with different concentrations of the extract for 1 h. The next step consisted in the stimulation with LPS (1  $\mu$ g/mL) for 18 h, and the effect of the tested extracts in the absence of LPS was also assessed. To the negative controls no LPS was added. Through this procedure it was possible to observe the occurrence of induced changes in NO basal levels. For that, the tested sample and LPS were dissolved in supplemented DMEM, and nitric oxide content was determined using a Griess Reagent System kit (Promega) containing sulfanilamide, NED and nitrite solutions. One hundred microliters of the cell culture supernatant were transferred to the plate and mixed with sulfanilamide and NED solutions, for 5–

10 min, at room temperature. Nitrite level produced was determined by measuring optical density at 515 nm, in a microplate reader, and then compared with the standard calibration curve. Dexamethasone (50  $\mu$ M) was used as positive control. Three independent experiments were carried out and the results were expressed in EC<sub>50</sub> values, i.e. the extract concentration that inhibited 50% of the NO production.

## 2.6. Evaluation of the anti-Candida activity

### 2.6.1. Yeast strains

Eight *Candida* strains belonging to the species *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* were used in this study. Four of them were from the American Type Culture Collection (ATCC), namely *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 750, while the other four were clinical isolates from the archive collection of the Laboratory of Medical Mycology, Universidade Estadual de Maringá, Brazil. In each experiment, *Candida* strains were sub-cultured overnight in SDA or SDB, at 37 °C, and then cellular density adjusted using a Neubauer chamber.

### 2.6.2. Effect on *Candida* planktonic cells

#### A) Minimal inhibitory concentration (MIC)

The antifungal activity of the *H. androsaemum* ethanol:water extract was determined by the broth microdilution method according to CLSI standard M27-A3 (Clinical and Laboratory Standards Institute, 2008), with some modifications as described for natural products (Dalben-Dota et al., 2010). For this, serial dilutions were performed at a ratio of 2, from 1:2 to 1:1024. In this way, the concentrations tested of the extract ranged from 0.02 to 12.5 mg/mL. The test was carried out in RPMI 1640 with L-glutamine (with sodium bicarbonate) and 0.165 M 3-(N-morpholino)-propanesulfonic acid (pH 7.2) as buffer, supplemented with 2% glucose, in 96-well flat-bottomed microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). After incubation at 37 °C for 24 h, MIC were determined by direct observation. After visualization of the resultant plate, the MIC values were determined as the antifungal concentration where there was no growth or even fungistatic effect, by comparison with the control (cells grown without extract). Then, the number of cultivable cells was assessed by the determination of number of colony forming units (CFUs), through several dilutions. After 24 h of incubation at 37 °C, the number of colonies formed was counted. The results were presented as total CFUs (log CFUs) and the experiments repeated in triplicate on three different occasions.

#### B) Time-kill curve determination

Time kill curves were performed for the four *Candida* reference strains, *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 750 with slight modifications to that previously described (Klepser, Wolfe, Jones, & Nightingale, 1997). Prior to testing, fungi were subcultured on SDA and the inoculum adjusted to  $1\text{--}5 \times 10^5$  yeasts/mL, in RPMI 1640 medium, using a Neubauer chamber. Then, each *Candida* strain suspension was grown in the presence of the *H. androsaemum* ethanol:water extract at a concentration of 25 mg/mL. The RPMI 1640 medium without plant extract was used as a positive control. Test suspensions were placed on a shaker and incubated at 37 °C. At predetermined time points (0, 1, 2, 4, 6, 8, 12, and 24 h), serial dilutions were performed on SDA for colony-forming units (CFU) determination. Following incubation at 37 °C for 24 h, the number of CFU was determined.

#### C) Cell membrane lesion determination

Flow cytometry analyses using propidium iodide (PI; Sigma) were performed to determine the effect of the *H. androsaemum* ethanol:water



extract on integrity of the *Candida* cells membrane. For that, *Candida* cells ( $10^6$  cells/mL) were incubated with the *H. androsaemum* ethanol:water extract at a concentration of 25 mg/mL for 4 h as described for time kill curve procedure. After this time, the cells were washed once with PBS and then stained with 1  $\mu$ g PI/mL for 30 min. The cells were rinsed again with PBS and finally resuspended in 200  $\mu$ L for flow cytometry analysis using EC800™ flow cytometer analyzer (Sony Biotechnology). Scattergram analyses were performed to evaluate morphological changes (size and complexity) and a total of 2000 events were accounted. The percentage of stained cells at FL3 (620 nm, red), representing cells with severe lesions of the membrane, was quantified.

### 2.6.3. Effect on *Candida* biofilms

#### A) Biofilm formation

In order to evaluate the effect of *H. androsaemum* ethanol:water extract on *Candida* species' biofilm formation, the extract was added at the beginning of their formation. For that, *Candida* cells were grown on SDA for 24 h at 37 °C, then inoculated in SDB and incubated for 18 h at 37 °C under agitation at 120 rpm. After incubation, cells were harvested by centrifugation at 3000g for 10 min, at 4 °C, and washed twice with 15 mL of phosphate-buffered saline (PBS; pH 7; 0.1 M). Volumes of 100  $\mu$ L of extract ( $2 \times$  final concentration) in RPMI 1640 medium were added to each well of microtiter plates. Subsequently, 100  $\mu$ L of standardized yeast cell suspension ( $2 \times 10^5$  yeasts/mL) was added and plates were incubated at 37 °C on a shaker at 120 rpm/min for 24 h to allow biofilm formation. The extract concentrations used in this study (12.5 mg/mL and 25 mg/mL) were based on our previous findings of antimicrobial susceptibility. Negative controls (200  $\mu$ L of only RPMI 1640 medium) were also included.

Biofilms were analyzed by CFUs determination. For that, after 24 h the total medium was removed and the biofilms washed once with 200  $\mu$ L of PBS. Then, the biofilms were scraped from the respective wells and the suspensions vigorously vortexed for approximately 2 min to disaggregate cells from the matrix (Alves et al., 2014). Serial dilutions were made in PBS, plated onto SDA and incubated for 24 h at 37 °C. The results were presented in terms of log CFUs.

### 2.7. Analysis of phenolic compounds

HPLC (Hewlett–Packard 1100, Agilent Technologies, Santa Clara, USA) was used to determine phenolic composition of the extract in the conditions previously described (Barros et al., 2012). Double online detection was carried out in a diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. Phenolic compounds were identified by comparing their retention time, UV–vis and mass spectra with those obtained from standard compounds, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve ( $1\text{--}100$   $\mu$ g/mL) for each available phenolic standard was constructed based on the UV signal: chlorogenic acid ( $y = 304.25x - 248.06$ ;  $R^2 = 0.999$ ); protocatechuic acid ( $y = 258.17x + 328.45$ ;  $R^2 = 0.998$ ); *p*-coumaric acid ( $y = 706.09x + 1228.1$ ;  $R^2 = 0.999$ ); epicatechin ( $y = 163.23x + 39.586$ ;  $R^2 = 0.999$ ); naringenin ( $y = 539.98x + 161.46$ ;  $R^2 = 0.999$ ); taxifolin ( $y = 224.31x + 148.41$ ;  $R^2 = 0.999$ ), and quercetin-3-*O*-glucoside ( $y = 336.36x + 358.06$ ;  $R^2 = 0.998$ ). For the identified phenolic compounds for which no commercial standard was available, the quantification was performed using the calibration curve of another similar compound. Analyses were carried out in triplicate and the results were expressed in mg per g of dried extract.

### 2.8. Statistical analysis

All statistical tests were performed using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., Armonk, NY, USA), and the means were compared using Tukey's honestly significant difference (HSD) multiple comparisons test and further coupled to Welch to verify the equality.

## 3. Results and discussion

### 3.1. Antioxidant activity

Free radicals, among them reactive oxygen species (ROS), play an important role as triggers in a wide variety of diseases, such as (neuro)degenerative and metabolic disorders, and even cancer (Fernandez-Panchon, Villano, Troncoso, & Garcia-Parrilla, 2008; Li et al., 2014). Numerous studies have been addressed to discover effective antioxidant compounds, their active concentrations and related modes of action, being currently considered one of the highest hot topics among the scientific community (Dato et al., 2013; Espín, García-Conesa, & Tomás-Barberán, 2007). In fact, a relation between premature aging, longevity impairment and oxidative stress status is known to exist, although despite the intensive investigation a general consensus was not established (Halliwell, 2012). On this field, phenolic compounds seem to be promissory antioxidant agents (Carocho & Ferreira, 2013; Fernandez-Panchon et al., 2008). Thus, and aiming to put forward alternative sources of phenolic compounds, the antioxidant potential of the *H. androsaemum* ethanol:water extract was evaluated using four different *in vitro* assays. The results are given in Table 1. The most relevant effect was observed in the LPI assay, followed by RSA, RP and CBI assays, which means that compounds present in the studied *H. androsaemum* extract act mainly as lipid peroxidation inhibitors. Valentão et al. (2004) also concluded that the hepatoprotective effects of *H. androsaemum* mainly derived from its strong ability to inhibit lipid peroxidation, which was in part attributed to its phenolic composition. The authors achieved a concentration-dependent (16, 62 and 250  $\mu$ g/mL) effective inhibition of lipid peroxidation, by measuring the levels of MDA equivalents. However, at the highest concentration, the obtained result was not so good as the positive control. Other authors also reported radical scavenging effects when assessing the antioxidant capacity of the *H. androsaemum* extract. Thus, Rainha et al. (2011) working with aqueous extracts from the aerial parts of different *Hypericum* species, concluded that *H. androsaemum* not only exerted a strong free

**Table 1**

*In vitro* antioxidant, cytotoxic and anti-inflammatory properties of the *H. androsaemum* ethanol:water extract (mean  $\pm$  SD).

	<i>H. androsaemum</i>	Positive control <sup>a</sup>
Antioxidant activity (EC <sub>50</sub> , $\mu$ g/mL)		
DPPH scavenging activity (RSA)	96 $\pm$ 3	41 $\pm$ 1
Reducing power (RP)	103 $\pm$ 1	41 $\pm$ 2
$\beta$ -carotene bleaching inhibition (CBI)	155 $\pm$ 5	18 $\pm$ 1
TBARS inhibition (LPI)	36 $\pm$ 1	23 $\pm$ 1
Cytotoxic activity (GI <sub>50</sub> values, $\mu$ g/mL)		
MCF-7 (breast carcinoma)	106 $\pm$ 11	0.91 $\pm$ 0.04
NCI-H460 (non-small cell lung cancer)	215 $\pm$ 13	1.0 $\pm$ 0.1
HeLa (cervical carcinoma)	205 $\pm$ 18	1.9 $\pm$ 0.1
HepG2 (hepatocellular carcinoma)	100 $\pm$ 7	1.1 $\pm$ 0.2
Hepatotoxicity (GI <sub>50</sub> values, $\mu$ g/mL)		
PLP2	>400	3.2 $\pm$ 0.2
Anti-inflammatory activity (EC <sub>50</sub> values, $\mu$ g/mL)		
NO production inhibition	179 $\pm$ 17	16 $\pm$ 1

<sup>a</sup> Positive controls, trolox, ellipticine and dexamethasone, were used for antioxidant, cytotoxic and anti-inflammatory activity assays, respectively. EC<sub>50</sub> values correspond to the extract concentration achieving 50% of antioxidant/anti-inflammatory activity or 0.5 of absorbance in reducing power assay. GI<sub>50</sub> values correspond to the extract concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2.

radical scavenging activity but also significant inhibitory effects against linoleic acid oxidation. In the same line, Valentão et al. (2002) evaluating the antioxidant potential of aqueous extracts prepared from *H. androsaemum* leaves, described their action as strong scavengers of reactive oxygen species, mainly superoxide radicals ( $IC_{50} = 2.9 \mu\text{g/mL}$ ). Similar radical scavenging effects of *H. androsaemum* aqueous extracts were also described by Hernandez et al. (2010) and Ramos et al. (2013); these latter also reported a significant ability to repair DNA damages. By comparing these results with those previously cited, it is clearly evident that the geographic origin of plant species and solvent extraction, among many other factors, exert a direct and determinant effect on its chemical composition, and consequently biological potential. Interestingly, all the previous cited authors correlated the observed biological effects with the chemical composition of *H. androsaemum* extract, namely with its abundance in phenolic compounds. Chlorogenic acids have been mentioned as the most abundant phenolic acids present in this extract (nearly 60%) (Hernandez et al., 2010; Rainha et al., 2011; Ramos et al., 2013; Valentão et al., 2002; Valentão et al., 2004), and therefore responsible for the observed antioxidant potential. Additionally, and not least important to emphasize, is that quercetin derivatives, although present in small extent, may also contribute for the final biological potential. Hence, *H. androsaemum* may be considered a source of bioactive molecules, especially phenolic compounds, with antioxidant potential and could act as reducing agents, hydrogen donors, singlet oxygen quenchers, peroxynitrites, superoxide, hydroxyl and peroxy radical's scavengers.

### 3.2. Cytotoxic activity in tumor and non-tumor cells

No toxicity was observed for non-tumor porcine liver cells (PLP2) at concentrations up to  $400 \mu\text{g/mL}$  of the studied *H. androsaemum* extract, in contrast with the high hepatotoxicity registered for the positive control ellipticine (Table 1). However, the extract was able to inhibit different human tumor cell lines: breast (MCF-7), non-small cell lung (NCI-H460), cervical (HeLa) and hepatocellular (HepG2) carcinoma, although relatively high concentrations were required (Table 1). Hepatocellular carcinoma cells ( $GI_{50} = 100 \pm 7 \mu\text{g/mL}$ ) were the most susceptible to the *H. androsaemum* extract, followed by breast ( $GI_{50} = 106 \pm 11 \mu\text{g/mL}$ ), cervical ( $GI_{50} = 205 \pm 13 \mu\text{g/mL}$ ) and non-small cell lung ( $GI_{50} = 215 \pm 13 \mu\text{g/mL}$ ) carcinoma cell lines. The obtained results could be due to the diversity and the occurrence of synergistic reactions between the different bioactive compounds present in the extract. In addition, it is also important to note that despite the obtained results were not highly promising when compared with the positive control ellipticine, this compound was also highly toxic for non-tumor cells at very low concentrations. As previously mentioned, the origin of the extract as well as the type of the extraction solvent may interfere with the final observed effects. For example, Xavier et al. (2012) reported the *in vitro* anti-proliferative and pro-apoptotic effects of *H. androsaemum* aqueous extracts in human colon carcinoma-derived cell lines, and described an  $IC_{50}$  concentration of  $65 \mu\text{g/mL}$ . Chlorogenic acid was the most abundant phenolic compound in the extract studied by these authors,  $54 \mu\text{g/mg}$  (Xavier et al., 2012), which is in agreement with the herein tested ethanol: water extract, where two chlorogenic acids, 5-O-caffeoylquinic acid and 3-O-caffeoylquinic acid were the majority compounds. Thus, it seems that chlorogenic acids could be the main responsible bioactive compounds for the observed cytotoxic potential. Notwithstanding, the authors clearly emphasized that the observed effect was not due to the presence of chlorogenic acid alone, once using a concentration 20 times higher than the one present in the extract, no positive effects were observed.

### 3.3. Anti-inflammatory activity

Inflammation is in the origin of numerous degenerative and metabolic disorders, among which the most tremendous is cancer.

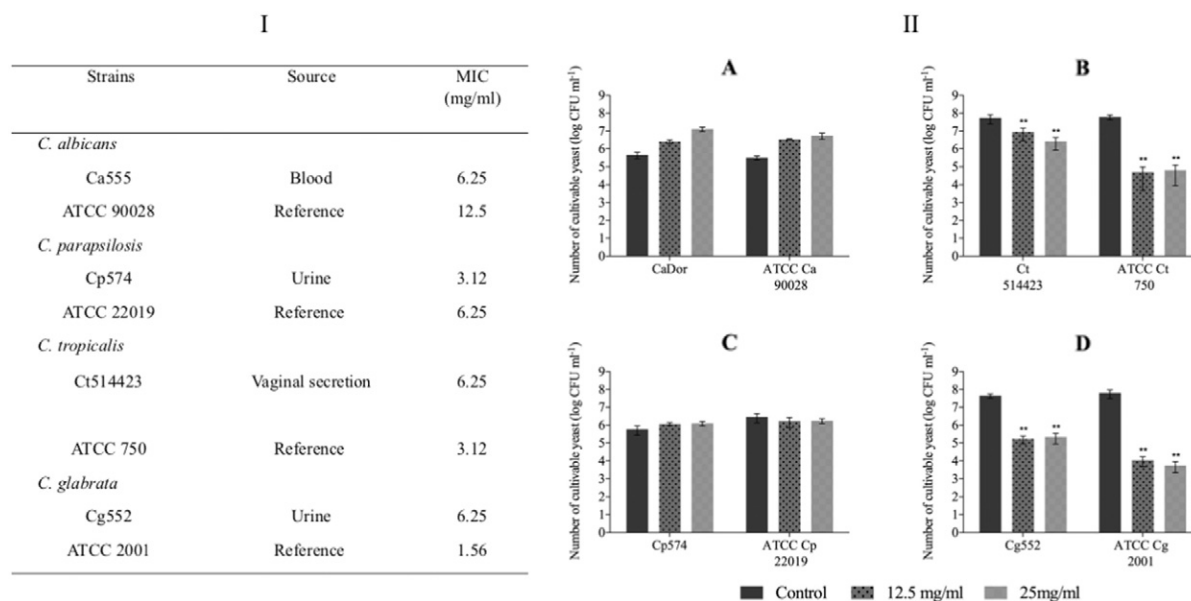
Notwithstanding, the inflammatory process is a very complex process, and in most of the cases the triggering factor cannot be identified, although free radicals overproduction might be one of the reasons (Aruoma, 2003; Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005). Thus, and considering the observed antioxidant potential of the *H. androsaemum* ethanol: water extract, their anti-inflammatory activity was also evaluated. A 50% inhibition of nitric oxide (NO) production was observed at  $178.78 \pm 16.60 \mu\text{g/mL}$  (Table 1). As far as we know, this would be the first report on the anti-inflammatory potential of *H. androsaemum* extracts. Considering the obtained results, and the fact that the phenolic composition may largely determine the observed effects, it is possible to infer that chlorogenic acids apart from displaying a significant antioxidant effect also contributes to ameliorate the inflammatory processes. In fact, it has been reported that substances with significant antioxidant properties, also exert anti-inflammatory effects. For example, Chen & Kang (2013) assessing the *in vitro* anti-inflammatory and antioxidant effects of *Capsicum annuum* L. (red pepper) observed a direct correlation between antioxidant and anti-inflammatory effects, and that these effects are directly dependent on its phenolic composition, namely in chlorogenic and *p*-coumaric acids, which were respectively, the most abundant phenolic compounds present. In addition, the authors also observed considerable differences between the extracts obtained from the pericarp, placenta and stalks, providing the last one the highest potential (Chen & Kang, 2013). On the other hand, Correia et al. (2005) aiming to evaluate the antioxidant potential of *Equisetum telmateia* Ehrh. and to determine its phenolic composition, observed that the content in proanthocyanidins was high, being then isolated for further evaluation. Moreover, and not least important to highlight was that proanthocyanidins were identified in this matrix for the first time by these authors, in particular propelargonidins, which probably explains the pronounced anti-inflammatory and antimicrobial effects of this plant, apart from its significant antioxidant potential (Correia, González-Paramás, Amaral, Santos-Buelga, & Batista, 2005). In our study, the contents of procyanidins were lower than those observed by Correia et al. (2005) in ethyl acetate fractions; but ethanol: water extracts are considered safer and "greener" options.

### 3.4. Anti-Candida activity

#### 3.4.1. Effect on *Candida* planktonic cells

##### A) Minimal inhibitory concentrations

As it can be observed in Fig. 1 (I), the extract was active against all the tested strains, with MIC values ranging from 1.56 to  $12.5 \text{ mg/mL}$ . Fig. 1 (II) shows the number of counts ( $\log \text{CFU/mL}$ ) for the susceptible *Candida* strains treated with the extract. The most pronounced fungistatic effect was observed against *C. glabrata* (Fig. 1(II) D) and *C. parapsilosis* (Fig. 1(II) C), for which the two assayed strains were sensitive with an observed effect that was directly related to the concentration tested. Variable effects were observed between each of the two strains in the other two *Candida* species considered. There are no doubts that both morphological and structural factors specific of each of the tested *Candida* species may explain the observed results, but it is also important to emphasize the different susceptibilities of *Candida* species, i.e. *Candida* species with high evidences of drug-resistance (such as *Candida tropicalis* strains) should normally display a low sensibility to the tested plant extract. In fact, this effect was observed, but the magnitude of growth inhibition was dependent to the concentration used. By comparing the obtained results with the positive control, fluconazole, they were not really promissory, being the MIC values to *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 750, respectively, 0.125, 8, 0.5 and  $0.5 \mu\text{g/mL}$ . However, it is important to point out that in this case a pure compound was used, while in the natural extract are present a rich pool of



**Fig. 1.** In vitro effects of the *H. androsaemum* ethanol:water extract on *Candida* strains planktonic cells. I) Minimal inhibitory concentrations (MICs values). II) Logarithm of number *Candida* species cells grown in the presence of increased concentrations of *H. androsaemum* during 24 h. (A) *Candida albicans*; (B) *Candida tropicalis*; (C) *Candida parapsilosis*; and (D) *Candida glabrata*. Error bars represent standard deviation. Statistical *p* value (represented by \* or \*\*) indicates concentrations that are significantly different from control. \**p* < 0.05; \*\**p* < 0.01.

phytochemicals with different potentialities, being therefore of the utmost importance to access its effect both individually and mixtures of several of those compounds, as well as its synergic potential with the conventional drugs. Furthermore, it is also crucial to note that the chemical composition of the teste plant extract largely determines its biological potential; thus, and considering that 5-*O*-caffeoylquinic acid followed by 3-*O*-caffeoylquinic acid were the most abundant phenolic compounds present in the *H. androsaemum* ethanol:water extract, the observed anti-*Candida* effect may be directly related by its abundance at the concentrations tested.

#### B) Time-kill curve determination

The killing activity of the *H. androsaemum* ethanol:water extract, plotted from log CFU/mL versus time (24 h), is represented in Fig. 2. Analysis of the time-kill curves showed an inhibitory effect against three of the tested species, *C. tropicalis*, *C. parapsilosis* and *C. glabrata*. At a concentration of 25 mg/mL of extract, a substantial time-dependent reduction in the number of cultivable cells was observed compared with the control group. The more pronounced effect was observed in *C. glabrata*, with a decrease  $\geq 99\%$  of the cells (2.2 logs) at 24 h, comparatively to control group. For the *C. tropicalis* and *C. parapsilosis* strains the reduction was about 90% (1.5 log) up to 12 h with a prominent re-growth after that time. However, no effects were observed on the pattern growth of *C. albicans*. The highest MIC value for the extract was also obtained in this species compared with *C. tropicalis*, *C. parapsilosis* and *C. glabrata*.

#### C) Cell membrane disruption

Flow cytometry analyses were used to investigate the effect of the *H. androsaemum* ethanol:water extract on the membrane integrity of fungal cells. PI is a nucleic acid-binding fluorescent probe commonly employed to evaluate the effect of drugs on cell membranes. Cells with severe membrane lesions leading to inherent loss of viability will internalize PI, resulting in an increase in red fluorescence (Pina-Vaz et al., 2001). The PI penetration in yeast cells treated with the extract is summarized in Fig. 3. After incubation periods of 4 h with 25 mg/mL of the extract, *Candida* cells were affected in a different manner.

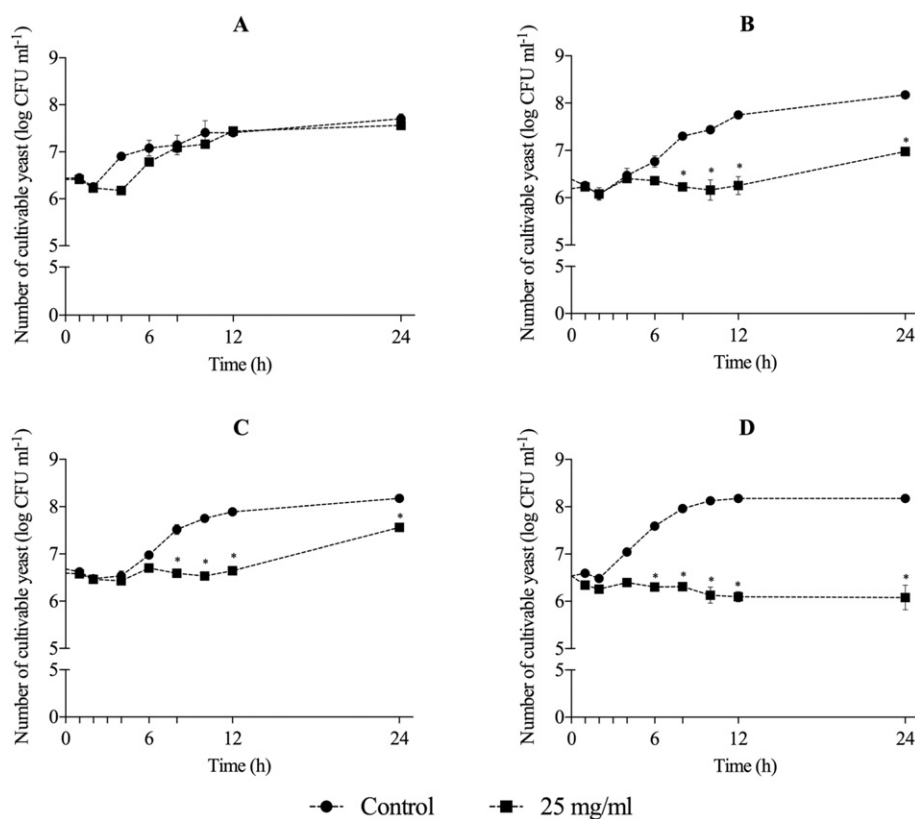
Interestingly, the extract was able to disintegrate cell membranes of *C. glabrata* and *C. tropicalis* more than to the other species, whereas no significant differences between *C. albicans*, *C. parapsilosis* and control were observed (Fig. 3). The obtained results are in accordance with the observations made both on the *Candida* cells growth inhibition (Fig. 1) and time-kill curve assays (Fig. 2). These data indicate that *C. tropicalis* and *C. glabrata* cells are more sensitive to the treatment with *H. androsaemum* evidencing high membrane permeability when compared to untreated cells. In addition, the involved mechanisms of the antimicrobial potential conferred by the main phenolic compounds present in *H. androsaemum* remain poorly characterized.

#### 3.4.2. Effect on *Candida* biofilms

Biofilms are structured communities that can cause serious medical problems, such as indwelling device-related infections. Recent data show that over 65% of all hospital infections derived from biofilms (Azevedo et al., 2014). Biofilm-associated *Candida* cells are resistant to a wide spectrum of available antifungal drugs (Ramage, Mowat, Jones, Williams, & Lopez-Ribot, 2009). This scenario has exacerbated by the increasing demand for safer and more effective antifungal therapies, and the search for new and better agents that target fundamental biological processes and/or pathogenic determinants (Khan, Ahmad, Cameotra, & Botha, 2014).

As presented in Fig. 4, *Candida* strains biofilm formation was attenuated in the presence of the *H. androsaemum* extract in the cases of *C. tropicalis* and *C. glabrata*, for which a reduction >90% of the cultivable cells was observed on the biofilms treated with the extract at concentrations of 12.5 and 25 mg/mL. *Candida glabrata* strains presented the highest biofilm reduction (~2.4 to 4.1 log units) (Fig. 4D), followed by *C. tropicalis* with a reduction around ~1 to 3 log units (Fig. 4B) compared with the control group. No significant differences were observed for *C. albicans* and *C. parapsilosis* biofilms in relation to the untreated biofilms (Fig. 4A and C). The results obtained for *C. glabrata* and *C. tropicalis* seem to be promising, once species NCAC exhibit more resistance to conventional antifungals. Moreover, *C. glabrata* are intrinsically resistant to fluconazole and other azoles, the most commonly used drugs to treat *Candida* infections.

Despite the weaker results in comparison with the positive controls used, the obtained data may also open new perspectives to discover more effective and safer antifungal drugs, considering that the majority



**Fig. 2.** Killing kinetics of the *H. androsaemum* ethanol:water extract against *Candida* species. A: *Candida albicans* ATCC 90028; B: *Candida tropicalis* ATCC 750; C: *Candida parapsilosis* ATCC 22019; D: *Candida glabrata* ATCC 2001. Standardized yeast cells suspensions were exposed to 25 mg/mL of *H. androsaemum* extracts. At determined time intervals, samples were serially diluted and plated for colony counts. Each data point represents mean result  $\pm$  standard deviation (error bars) from three experiments. Statistical *p* value (represented by \*) indicates concentrations that are significantly different from control. \**p* < 0.05.

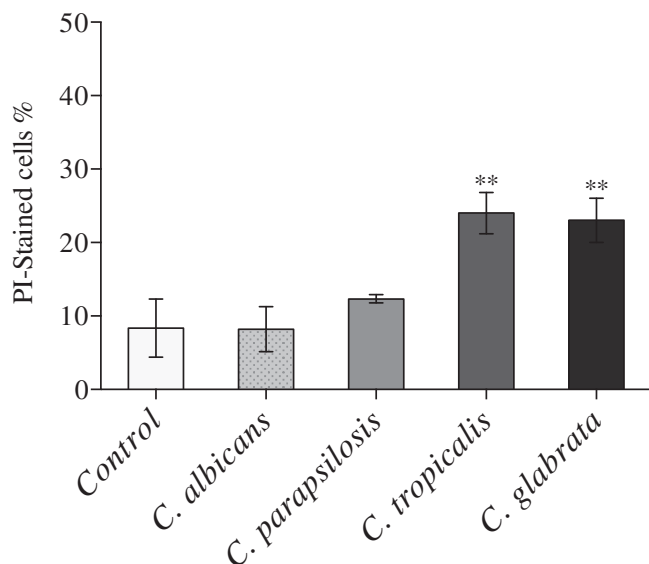
of them derives from plants, and then are synthesized in large scale. For example, Pendota, Aderogba, Ndhlala, & Staden (2013) evaluated the antifungal potential of *Buddleja salviifolia* (L.) Lam. leaf extracts and its isolated compounds, and observed that MIC values obtained for hexane,

dichloromethane, ethyl acetate and butanol extracts, as also for individual compounds were higher than those obtained for the positive control. However, the obtained MIC values for the individual compounds were lower than those for the whole extracts (Pendota et al., 2013). It means that a wide variety of metabolic and biochemical reactions may occurs between the different phytochemicals present in natural matrices, some of them improving the final biological effect while other ones exert an antagonistic effect.

### 3.5. Phenolic compounds

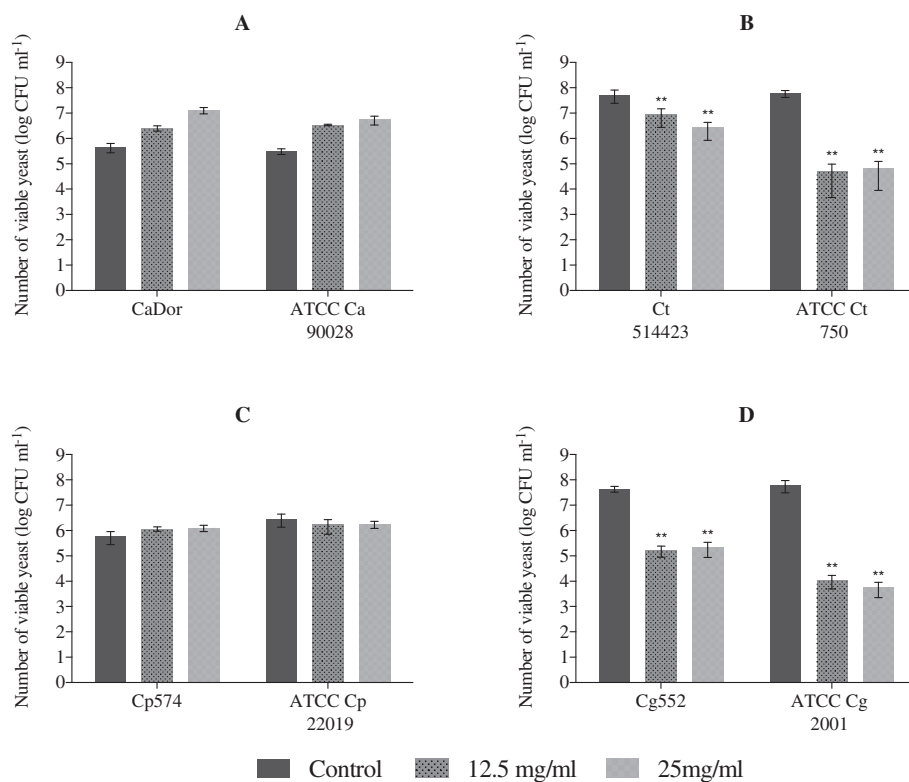
The chromatographic profile of the *H. androsaemum* ethanol:water extract recorded at 280 nm is shown in Fig. 5, and peak characteristics, tentative identities and phenolic compounds quantification are presented in Table 2. Compounds 2 (protocatechuic acid), 4 (5-*O*-caffeoylquinic acid), 6 (epicatechin), 11 (taxifolin), 12 (quercetin-3-*O*-glucuronide) and 13 (quercetin-3-*O*-glucoside) were positively identified by comparison with authentic standards, as also by their MS fragmentation pattern, retention time and UV-vis characteristics. 5-*O*-Caffeoylquinic acid and quercetin derivatives have been reported in methanol extracts from the aerial parts (Rainha et al., 2011) and leaves (Porzel et al., 2014; Valentão et al., 2003) of *H. androsaemum*, and in aqueous extracts from the leaves (Valentão et al., 2002; Valentão et al., 2004).

Compound 1 ([M-H]<sup>-</sup> at *m/z* 353) was identified as 3-*O*-caffeoylquinic acid based on its fragmentation pattern, yielding a base peak at *m/z* 191 (deprotonated quinic acid) and an ion at *m/z* 179 [caffeic acid-H]<sup>-</sup> with an intensity of 47% of the base peak, as reported by Clifford, Johnston, Knight and Kuhnert (2005, 2003), and Clifford, Johnston, Knight, and Kuhnert (2003). Similarly, compound 3 ([M-H]<sup>-</sup> at *m/z* 337) was tentatively identified according to its MS<sup>2</sup> fragmentation as 3-*O*-*p*-coumaroylquinic acid (Clifford et al., 2003; Clifford et al.,



**Fig. 3.** Effect of the *H. androsaemum* ethanol:water extract on the *Candida* species membrane. The percentage of PI-stained cells was analyzed by flow cytometry with and without exposure to *H. androsaemum* extracts for a period of 4 h. Statistical *p* value (represented by \*\*) indicates concentrations that are significantly different from control. \**p* < 0.01.





**Fig. 4.** Effect of the *H. androsaemum* ethanol:water extract on *Candida* strains biofilm. Logarithm of number of colony form units (CFUs) of different *Candida albicans* (A), *Candida tropicalis* (B), *Candida parapsilosis* (C) and *Candida glabrata* (D) biofilm strains, treated with different concentrations of the *H. androsaemum* extracts. *Candida* cells were allowed to forming biofilm for 24 h with extract at 37 °C. Controls: biofilm grown in RPMI medium in the absence of extract. Error bars represented in graphics indicate the standard deviation; \*\* correspond to  $p < 0.01$ .

2005). These compounds have been already reported in aqueous (Valentão et al., 2002; Valentão et al., 2004) and methanolic (Porzel et al., 2014; Valentão et al., 2003) extracts from leaves and in aerial parts (Rainha et al., 2011) of *H. androsaemum*.

Compounds 5, 7, 8 and 10 showed UV spectra with  $\lambda_{\max}$  280 nm, characteristic of proanthocyanidins. Peaks 5 and 10 presented pseudomolecular ions corresponding to (epi)catechin dimers ( $[M-H]^-$  at  $m/z$  577) and compounds 7 ( $[M-H]^-$  at  $m/z$  865) and 8 ( $[M-H]^-$  at  $m/z$  1153) to a trimer and a tetramer, respectively. The positive identification in the extract of epicatechin as the only flavan-3-ol monomer would suggest an epicatechin-based identity for those peaks. Thus, the compounds might correspond to the B-linked epicatechin series, i.e., procyanidin dimer B2 (epicatechin-4,8-epicatechin, peak 5), trimer C1

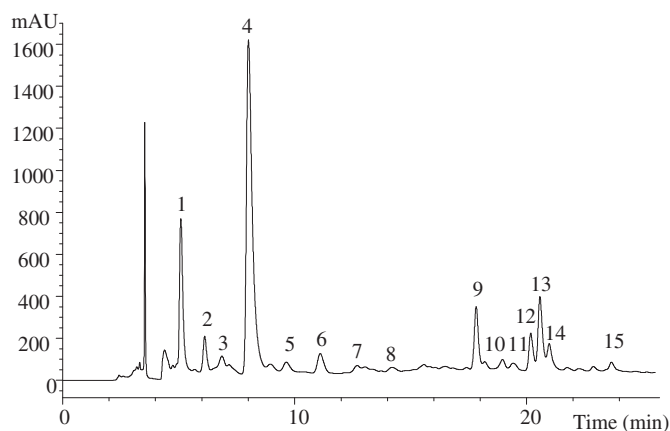
(epicatechin-4,8-epicatechin-4,8-epicatechin, peak 7) and the (4,8)-linked epicatechin tetramer (peak 8), whereas the late elution of peak 10 suggests a (4,6) linkage (i.e., epicatechin-4,6-epicatechin; dimer B5). These identities would also be coherent with their expected relative order of elution (Santos-Buelga, García-Viguera, & Tomás-Barberán, 2003). This identification differs from the one recently reported by Ramalheite et al. (2016), who indicated the presence of (+)-catechin and related proanthocyanidins in methanol extracts obtained from the aerial parts of this plant.

Compounds 14 ( $[M-H]^-$  at  $m/z$  463) and 15 ( $[M-H]^-$  at  $m/z$  433) presented UV spectra ( $\lambda_{\max}$  354–356 nm) and the production of a unique fragment ion at  $m/z$  301 ([quercetin- $H$ ] $^-$ ), which allowed their identification as quercetin-*O*-hexoside and quercetin-*O*-pentoside, respectively. Finally, compound 9 ( $[M-H]^-$  at  $m/z$  449) was tentatively identified as eriodictyol-*O*-hexoside, based on its UV spectrum and the product ion observed at  $m/z$  287. No further conclusion about the nature and position of the substituting sugar of these compounds can be made from the obtained LC-DAD-MS data. Similar quercetin glycosides have been indicated in aqueous (Valentão et al., 2002; Valentão et al., 2004) and methanol (Porzel et al., 2014; Rainha et al., 2011; Valentão et al., 2003) preparations of the plant, whereas, to the author's best knowledge, the presence of eriodictyol-*O*-hexoside has not been previously reported.

Overall, 5-*O*-caffeoylquinic acid followed by 3-*O*-caffeoylquinic acid were the majority phenolic compounds present in the *H. androsaemum* ethanol:water extract, and they should account for the observed antioxidant, cytotoxic, anti-inflammatory and anti-*Candida* activities.

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**Fig. 5.** Phenolic compounds profile of the *H. androsaemum* ethanol:water extract recorded at 280 nm.



**Table 2**  
Retention time (Rt), wavelengths of maximum absorption in the UV–vis region ( $\lambda_{\text{max}}$ ), mass spectral data, identification and quantification (mean  $\pm$  SD) of phenolic compounds in the *H. androsaemum* ethanol:water extract.

Compound	Rt (min)	$\lambda_{\text{max}}$ (nm)	Pseudomolecular ion [M-H] <sup>−</sup> (m/z)	MS <sup>2</sup> (m/z)	Tentative identification	Quantification (mg/g extract)
1	5.1	328	353	191(100),179(47),161(5),135(38)	3-O-Caffeoylquinic acid	11.6 $\pm$ 0.1
2	6.1	260,294sh	153	109(100)	Protocatechuic acid	4.29 $\pm$ 0.001
3	6.9	312	337	191(25),173(10),163(100),119(65)	3-O-p-Coumaroylquinic acid	0.88 $\pm$ 0.01
4	8.0	328	353	191(100),179(3),161(6),135(4)	5-O-Caffeoylquinic acid	40.1 $\pm$ 0.1
5	9.0	280	577	451(25),425(50),407(100),289(63),287(8)	Procyanidin B2	5.6 $\pm$ 0.1
6	11.1	280	289	245(18),203(11),187(4),161(7),137(8)	(-)-Epicatechin	6.9 $\pm$ 0.2
7	13.1	280	865	739(4),713(3),577(8),575(4),425(19),407(20),289(23),287(8)	Procyanidin C1	3.4 $\pm$ 0.2
8	14.2	280	1153	865(9),863(12),577(25),575(42),289(5),287(9)	Epicatechin tetramer	4.4 $\pm$ 0.2
9	17.9	286,336sh	449	287(100)	Eriodictyol-O-glucoside	3.7 $\pm$ 0.2
10	19.0	280	577	451(67),425(61),407(100),289(82),287(14)	Procyanidin B5	4.8 $\pm$ 0.4
11	19.4	288,340sh	303	285(100),199(12),177(8),150(15),125(35)	Taxifolin	3.4 $\pm$ 0.1
12	20.2	350	477	301(100)	Quercetin-3-O-glucuronide	4.71 $\pm$ 0.01
13	20.6	354	463	301(100)	Quercetin-3-O-glucoside	10.69 $\pm$ 0.02
14	21.0	354	463	301(100)	Quercetin-O-hexoside	4.41 $\pm$ 0.04
15	23.7	356	433	301(100)	Quercetin-O-pentoside	1.33 $\pm$ 0.02
					Total phenolic acid derivatives	56.9 $\pm$ 0.2
					Total flavonoids	53 $\pm$ 1
					Total phenolic compounds	110 $\pm$ 0.4

Extraction yield: 33%. sh - shoulder.

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